Fabrication and Characterization of a Silk-HA Based Scaffold for Multi-compartmental B-L-B Graft

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National Institute of Technology, Rourkela

Certificate

This is to certify that the report entitled, "**Fabrication and Characterization of a Silk-HA Based Scaffold for Multi-compartmental B-L-B Graft**", submitted by Miss. **Sasmita Majhi**, Roll no.: **109BM0009**, B.Tech-8th semester, Department of Biotechnology & Medical Engineering, National Institute of Technology, Rourkela (Deemed University) is an authentic work carried out by her under my supervision and guidance.

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Sasmita Majhi

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ABSTRACT

Tissue engineering being a central forum for ground breaking scientific research and developments in biomedical applications caters to design and fabrication of new tissues by development of biodegradable substitutes for functional restoration & regeneration of impaired organs. The key to success in tissue engineering is customized and precisely optimized scaffolds specific to the tissue type. With the highest demand in musculoskeletal tissue engineering, it's a challenge to construct a complex scaffold for incorporating interface organ to bridge ligament/tendon with bone to replace injured ligaments beyond repair. Such a scaffold must act as a template for tissue formation providing a 3D platform to the seeded cells in addition to providing enough mechanical strength required at soft to hard tissue interface to construct a Bone-Ligament-Bone graft. In the current project, a scaffold for bone compartment of a multi-compartmental B-L-B graft was fabricated by knitting silk fibroin as core material to impart mechanical strength. Hydroxyapatite (HA) being known for its osteoconductive and osteoinductive properties, was integrated on the surface of silk fibroins by electrospinning along with the biodegradable polymer, PCL(Poly caprolactone). The fabricated scaffold was characterized for mechanical properties like biodegradability, stiffness, porosity in addition to biocompatibility features like cell adhesion efficiency, cell proliferation after seeding fibroblasts. The nano-microscaffold showed the capability to support growth and proliferation of seeded fibroblast cells and an average yield stress of 38±0.18 kg/cm2. It was concluded that the fabricated scaffold can be effectively used for bone tissue engineering that needs further confirmation using bone cells.

Keywords: *Silk, Scaffold, Knitting, Electrospinning, Hydroxyapatite, PCL, Fibroblast.*

CHAPTER -1

INTRODUCTION

1. Introduction

Recently a new process to create a hard tissue (bone)-soft tissue (cartilage or ligament) bilayered scaffold was developed. Because of sports-related injuries and aging population musculoskeletal disorders have been converted into one of the major health concerns. Bone repair typically involves the use of allografts or autografts, ligament repair often uses patellar tendon, whereas cartilage repair requires use of cartilaginous grafts or joint resurfacing. In most cases of musculoskeletal disorder, total joint replacements are done using prosthetics. Because of insufficient supply of donor tissue, this technique cannot always be practical. As well as obtaining tissue from other source poses the risk of immunorejection and disease transfer. Tissue engineering may be a way to evade the limitations of existing therapies by applying the principles of engineering and biology in solving the problems associated with partial or whole organ transplantation. One of the approach is to use three-dimensional porous, biodegradable polymeric scaffolds, which provides support for the in growth of new tissue as the scaffold degrades devices have been used. Yet another approach is to culture the cells on a preformed three-dimensional scaffold and transplant the cell-polymer construct into the patient.

Electrospinning is considered to be one of the most promising processes in the field of nanotechnology due to its simplicity, low cost, high productivity, reproducibility and its potential to be scaled up to the industrial scale .The method involves the application of a high voltage electric field to draw very thin fibres from a polymeric fluid stream (solution or melt) delivered through a millimeter-scale nozzle. Electrospinning technique depends on various processing parameters such as solution properties (e.g., viscosity, surface tension and conductivity) and processing parameters (e.g., electric field strength, solution flow rate, needle diameter and distance between needle tip and ground collector).

Nano fiber is defined as the fiber having at least one dimension in nanometer range. Nano fiber is used for a wide range of medical applications for drug delivery systems, scaffold formation, wound healing and widely used in tissue engineering, skeletal tissue, bone tissue, cartilage tissue, neural tissue ,ligament tissue, etc.

CHAPTER -2

LITERATURE REVIEW

2. Literature Review

2.1 Musculoskeletal system

2.1.1 Soft tissue

During embryogenesis, gastrulation occurs in the 3rdweek of development. This process establishes all three germ layers in the embryo. The ectodermal layer gives rise to organs and structures that are in contact with the outer world, e.g.; central and peripheral nervous system, sensory epithelium, epidermis, and mammary gland (1). The mesodermal layer forms the dermis and subcutaneous layers of the skin, connective tissue, cartilage, bone, muscle, blood cells, and endothelium. Regarding the ECM in developing tissues, two major alterations occur: the components of the ECM change, and the cellular reactivity to the ECM components change. The ECM and cell surfaces interact functionally and this plays an intense role in the development and maintenance of a number of cells and tissues. Hence, the ECM is also instructive, or informational, and greatly influences cell behaviour. ECM is mainly composed of collagen, glycoproteins and proteoglycans,. Different ECMs contain different elements which define their tissue specificity (2).

2.1.2 Enthesis Composition and Structural Organization

An enthesis is the specialized arrangement of connective tissue that comprises the attachment of a muscle to a bone while permitting the muscle's fibres to be organised in appropriate pennation. An enthesis may be categorised into a fibrous enthesis or a fibro-cartilaginous according to (Benjamin *et al*.) based on the characteristics of the tissue at the bone-tendon interface location. A fibrous enthesis is composed with mainly dense fibrous connective tissues and can be further divided into two categories – periosteal and bony, depending on the region to which the tendon attaches. On the other hand, a fibro-cartilaginous enthesis appears in the area which is subject to compression and shows two more additional zones between connective tissue and bone – uncalcified fibrocartilage and calcified fibrocartilage. Entheses are mostly the sites of musculoskeletal overuse injuries that include tennis elbow and jumper's knee.

2.2 Properties of Soft tissue

Soft biological tissues mostly encounter high range of diversity in its mechanical properties. These organic tissues are generally characterized by complex mechanical behaviour. They used to show non-linear, viscoelastic, and anisotropic behaviour. They often have a layered or complicated structure. Inhomogenity in mechanical properties is due to varied positions in material. The perfusion of the organs and their constituting tissues are important while regarding the elastic properties.

2.2.1 Mechanical Property of Load Bearing Tissue

Soft biological tissues have mainly two sources of elasticity. The first source of elasticity is due to changes in internal energy whereas the second one is due to changes of entropy. Change of entropy occurs in tissues due to changes of orientation or waviness of fibers during loading or unloading.

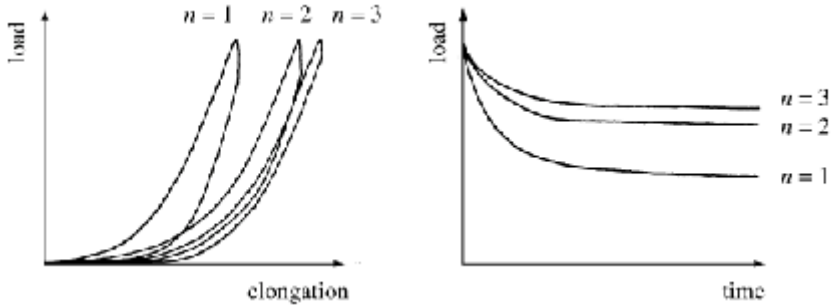


Figure 2.1 - Load-elongation and load-time diagram

With repeated loading cycles the load-deformation curves shift to the right in a load-elongation diagram and the hysteretic effects diminish. In a load-time diagram the load-time curves shift upwards with increasing repetition number. Repeated cycling, eventually leads to a steady state at which no further change occurs until the cycling routine is changed. And this state the tissue is considered to be preconditioned. Changes in the lower or upper limits of the cycling process require new preconditioning of the tissue. Internal changes in the structure of the tissue causes preconditioning. Hysteresis, non-linearity, and preconditioning are common properties of all soft tissues. The hysteresis in the stress strain relationship clearly shows the viscoelastic behaviour of soft biological tissue. In a viscoelastic material the history of strain affects the actual observed stress. Loading and unloading occur on different stress-strain

paths leading to hysteresis. The hysteresis of most biological tissues is assumed to show only little dependence on the strain rate within several decades of strain rate variation. This insensitivity in strain rate over several decades is not compatible with simple viscoelastic models like: single spring and dashpot element. For such simple viscoelasticity approach the material model will show a maximum hysteresis loop at a certain strain rate whereas all other strain rates will show a smaller hysteresis loop. A model having a discrete number of spring-dashpot elements produces a discrete hysteresis spectrum with maximum dissipation at discrete strain rates. By choosing the relaxation times of the different elements adequately a series of spring-dashpot elements might be used as an approximation to a continuous relaxation spectrum. Living tissues often show viscoelastic behaviour as shown qualitatively in the figure below.

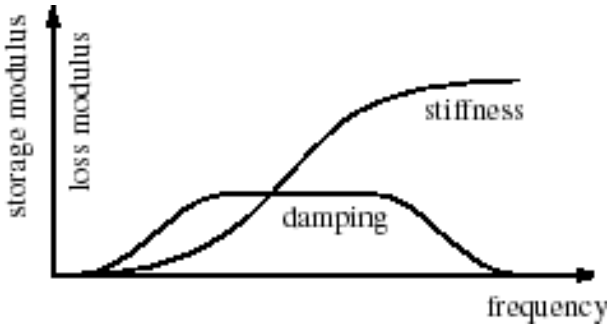


Figure 2.2 - Viscoelastic behaviour of living tissue

In the above figure the viscoelastic material properties are characterized by storage modulus and loss modulus. With a series of spring-dashpot elements arbitrary viscoelastic material properties can be modelled.

2.2.2 Properties of Hard tissue

In bone, the main determinant of mechanical properties is the amount of mineral in the tissue. Presence of more mineral displaces water making the bone becomes stiffer, but at the same time becomes more brittle.

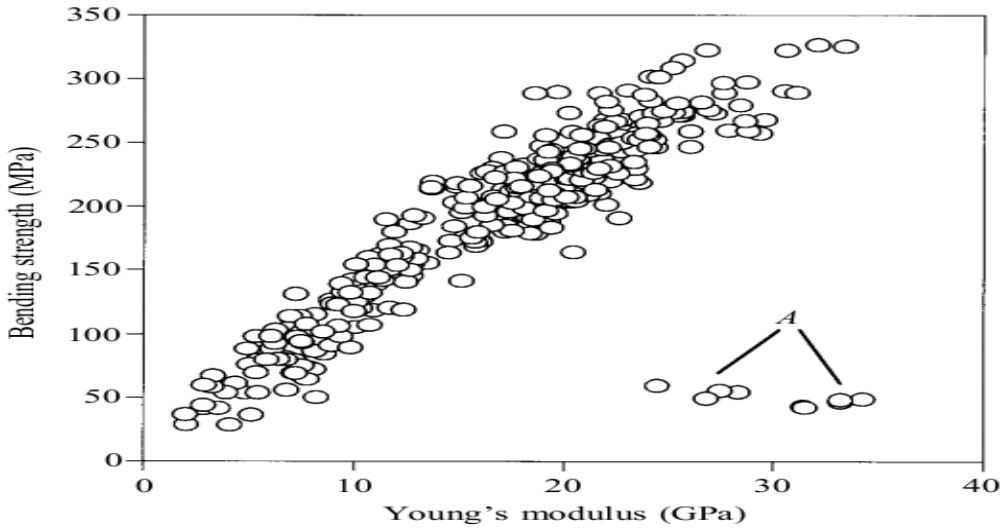


Figure 2.3 - Mechanical properties of mineralised tissue

Fig. shows the relationship between the stiffness (Young's modulus) of bone and its bending strength. These values come from bone specimens from a wide variety of amniote species, each point representing the value for a single specimen. Apart from the specimens labelled 'A', there is clearly a very strong, almost linear, relationship between Young's modulus and strength, with the strength values being approximately 1/100 of the stiffness values.In 1991, Currey *et al.* demonstrated that the failure of bone in bending is determined by the strain to which it is subjected**.** This relationship seems to be true whether differences in modulus are determined by differences in mineralisation and/or by differences in porosity. That is to say, it is the strain in the outermost fibres of a specimen that will determine whether a specimen breaks when subjected to a particular bending moment, no matter what causes it to have these high strains.

2.3 Tissue engineering & organogenesis

The basic concept of tissue engineering includes a scaffold that provides a architecture on which seeded cells can organize and develop into the desired organ or tissue prior to implantation. The scaffold provides an early biomechanical contour for the replacement tissue until the cells produce an adequate amount of extracellular matrix. During the formation and deposition of the new matrices, the scaffold is degraded eventually leaving a vital organ or tissue that restores, maintains, or improves tissue function(11).

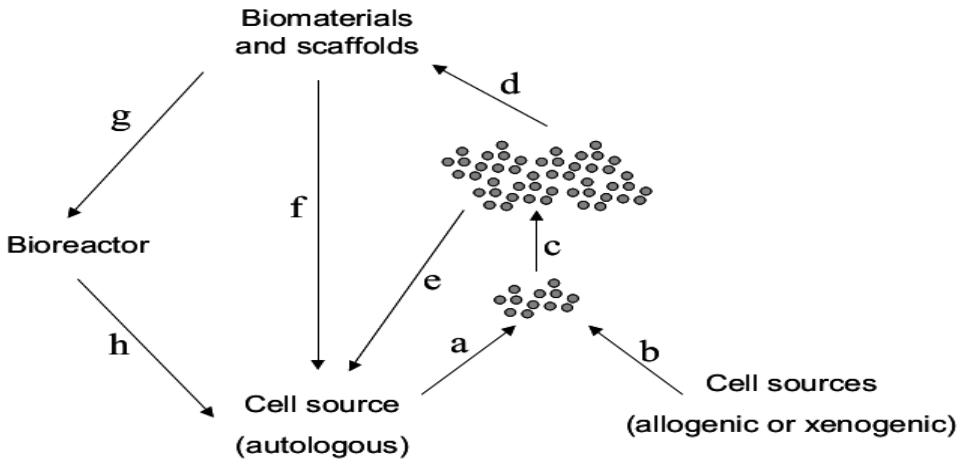


Figure 2.4 - Tissue growth in bioreactor

Biomaterials, cell sources, growth factors for cell and bioreactors are four components for tissue engineering. In this part, these four components are reviewed for tendon and ligament tissue engineering.

2.3.1 Biomaterial and Scaffold

There are several requirements govern the choice of materials for tendon and ligament tissue engineering. Firstly, the material must be non-toxic and biocompatible (12). The material must not cause abnormal responses in local tissues surrounding the implant and should not produce toxic or carcinogenic effects. Secondly, the material should avoid infection, so that the material should be sterile-able before seeding the cells onto the scaffold, and should stay sterile prior to implantation. The sterilization technique should not alter the mechanical and physical properties of the material. Finally, the scaffold material should provide some mechanical integrity initially to replace the function of the lost tendon or ligament before the scaffold degrades and is replaced with new extracellular matrix. Tendon and ligament have extremely hierarchal organized collagen structure, hence the requirement of the scaffolds for tendon and ligament repair is that the scaffolds need the similar micro and macro morphology as tendon and ligament.

2.3.2 Cells

Fibroblast: There are two types of tendon fibroblast cells in tendon, tenocytes and tenoblasts. And fibroblast is major cell type in ligament. Some studies have been done using fibroblasts for tendon and ligament repair in animal models (13).

Stem cells: Stem cells are very attractive cell sources for tendon tissue engineering. Stem cells are undifferentiated cells and have the ability to self-renew and differentiate to one or more types of specialized cells. Stem cells can be classed to two types: embryonic stem (ES) cells and adult stem cells. Sources of ES cells: blastocysts and fetal tissue. These have got importance in the field of tissue engineering and regenerative medicine because they have the potential to produce most types of cells in the body (14). Although the potential of ES cells in tissue engineering is vast, there are many problems must be overcome. For example, human ES cells may be contaminated by animal cells or proteins. It is reported that nonhuman protein expressed by human ES cell lines grown on animal feeder layers (15). Other problems such as immunorejection and tumorigenesis are also obstacles for the clinical applications of ES cells.

2.3.3 Growth Factor

Growth and differentiation of ligament and tendons tissue are influenced by various growth factors: basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor (IGF)-1, and members of the family of TGF-β/bone morphogenetic proteins (TGF-β/BMPs).

2.3.4 Bioreactor

Bioreactors are essential for meeting the complex requirements of in vitro engineering of functional skeletal tissues. Cell proliferation and collagen synthesis is reported for chicken tendon cultured in vitro, undergoing cyclic tension for 14 days. Two major strategies may be applied for tendon tissue engineering. One is the in vivo approach of tendon engineering involving in situ delivery of cells, biomolecules, hydrogels or scaffolds to boost the regeneration and healing process to deal with the repair of small defects and the induction of tissue self-regeneration(16) .The other is the in vitro tissue engineering involving the growth of tendon-like tissue structure outside of the body and the implantation into the defect. This strategy is more concerned with larger defects or tissue replacement.

2.4 Bone

Bone is a dynamic, highly vascularised tissue having the unique capacity to heal and remodel injuries as well as its capacity to rapidly mobilize mineral stores, formulate it as the ultimate smart material. Its main function is to provide structural support for the body. The selection of the most appropriate material to produce a scaffold to be used in bone tissue engineering applications is a very important step towards the construction of a tissue engineered product, since its properties will determine, to a great extent, the properties of the scaffold (16)**.**They can be from natural (e.g., coralline hydroxylapatite (HA)) origin or synthetic such as synthetic HA or β-tricalcium phosphate (β-TCP).Because of their fascinating properties, osteoconductivity and osteoinductivity, they have been considered for bone tissue engineering. Several works have revealed good results concerning bone regeneration by using ceramics with or without bone marrow cells. In 2001,R.Quarto *et al.* encompassed major drawbacks of these materials.

To begin with they are brittle and present a low mechanical stability, which prevents their use in the regeneration of large bone defects. Because of osteoclastic activity, their degradation rates are difficult to expect. This could prove a problem because if it degrades too fast it will compromise the mechanical stability of the construct, which is low by itself. At the same time, this would dramatically lead to as .In 2001,C.S.Adams *et al.*, verified that increment in the extracellular concentrations of Ca and P, can cause cellular death. There are also alternative biodegradable polymers, which are supposed to be the ideal materials for bone tissue engineering. These can be divided in two groups: natural and synthetic.

Natural biodegradable polymers are those obtained from natural sources.These include: collagen, fibrinogen, chitosan, starch, hyaluronic acid (HA), and poly (hydroxybutyrate). The main advantages of these materials are their potential for low immunogenic response, their impending bioactive behavior and the capability of interacting with the host's tissues. Synthetic biodegradable polymers are generally used in biomedical engineering field. As the processability and chemical versatility of those polymers varies according to their structure and nature, and hence a direct relation cannot be established with the natural polymers. The most widely used are poly (α-hydroxyacids), poly(e-caprolactone), poly(propylenefumarates), poly(carbonates), poly-(phosphazenes),and poly(anhydrides).

2.4.1 Cells for Bone Tissue Engineering

Osteoblasts

Osteoblasts are generally isolated from biopsies taken from the patients (autologous), followed by limited expansion in vitro because of their non-immunogenicity. However this method has got several limitations: it takes a lot of time, comparatively few cells are available after the dissociation of the tissue and their spreading out rates are relatively low, restricting the number of cells to be seeded on the scaffolds. And in certain bone related diseases osteoblasts may not be appropriate for transplantation as their protein expression profile is under the expected values (18).

Embryonic Stem Cells

ES cells inhabit in the inner cell mass of the blastocyst. Later on it was found that after being transferred to early mouse embryos ES cells could give rise to all somatic cell types of the embryo, including the germ line(20).Up to now it was reported the isolation of ES cells from rodents primates and human beings.

Adult Stem Cells

Adult stem cells (ASCs) reside in the fully differentiated or adult tissues. ASCs were found in the bone marrow, periosteum, muscle, fat, brain, and skin. In the bone tissue engineering field there has been a special interest in the stem cells located in the bone marrow, known as Mesenchymal Stem Cells (MSC). The idea that bone marrow contained some kind of osteogenic precursor cells started in 1963, showed that by implanting pieces of bone marrow beneath the renal capsule, it was possible to acquire an osseous tissue. After this Friedenstein and co-workers revealed a series of in vivo studies in which it was shown the possible existence of osteogenic stem cells in the bone marrow.In 1968,A.J.Friedenstein *et al.*, developed a method to isolate fibroblast-like cells from the marrow to understand the nature and source of these cells. Later he coined the term colony-forming units fibroblastic (CFU-F) to describe these cells that were fibroblastic, non-phagocytic and clonogenic in nature.

2.4.2 Growth Factors

Like other tissues bone tissue also possess a surplus of growth factors.These includes: bone morphogenetic proteins (BMPs), transforming growth factor beta (TGFb), fibroblast growth factors (FGFs), insulin growth factor I and II (IGF I/II), and platelet derived growth factor (PDGF) are the most common and those that have realistically been proposed for bone tissue engineering applications(21).

2.4.3 Bioreactors

Bone is considered as highly structured mechanically active 3D tissue. The biological environment of osteoblasts is thus derived from a dynamic interaction between active cells experiencing mechanical forces and a continuously changing 3D matrix architecture(22). In order to develop tissue engineered products in vitro it is thus needed to develop adequate cell/scaffold culture systems that mimic the dynamics of the in vivo environment. Till date two systems have been normally used, spinner flasks and rotating wall vessel reactors (RWVR).

CHAPTER -3

MATERIALS

AND

METHODS

3. Materials and Methods

3.1 Procurement of degummed silk

Degummed silk fibres (Tasar) were procured from Central tasar research and training institute, Central silk board Jharkhand, INDIA).

Figure 3.1 - Degummed silk

Figure 3.2 - Knitting machine

3.2 Knitting of silk fibre

Silk fibres were wound into yarns. Yarns were made into varied turns (3, 5, 8, 12). Scaffolds were prepared from all respective turns of silk. According to instructions in manual the tension in the machine was adjusted between 0-4 to carry out the knitting with desired pore

size. The steps were followed to arrange the fibre in the machine for knitting. Scaffolds dimension were adjusted to (5cm*2cm).Hence the required needles (9 nos.) were used. To start with knitting the fibre bundle was kept perpendicular under K-Carriage arm. During knitting the pressure was maintained through out to avoid any knot in scaffolds or loosening during preparation. The scaffold was given tension using clip to keep it elongated during knitting. Scaffolds were then arranged inside metal strips to keep them stretched.

Figure 3.3 - Knitted silk scaffold

3.2.1 Morphological characterization of knitted scaffold

Scanning Electron Microscopy (SEM) Analysis:

The morphology and structure of the knitted fibres can be analyzed under optical scanning electron microscope. A JEOL JSM- 6480LV SEM was used in the experiment for characterization of fibres at an accelerating voltage of 15 KV. Scaffold samples were cut into small pieces with the help of scissor. Small scaffolds were mounted with the help of carbon tape on the sample holder. Each sample was then coated with a thick layer of platinum by a JEOL JFC -1600 auto fine coater .And the operating conditions were maintained at 20 mA for 90 seconds.

3.2.2 Determination of Porosity of knitted Scaffold

The pore size was obtained from SEM images of knitted scaffold using the Image J program.

3.2.3 Mechanical testing of knitted scaffolds

Tensile strength of scaffold was tested using TA-HDPlus Texture Analyser.

Figure 3.4 - TA-HDPlus Texture Analyser.

3.3 Biodegradability test

Preparation of 1X Phosphate Buffered Saline (PBS Buffer):

8g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4, 0.24g of KH_2PO_4 were dissolved in 800ml distilled H_2O. P^H was adjusted to 7.4 with HCl. The volume was adjusted to 1L with additional distilled H_2O.PBS was sterilized by autoclaving. Scaffolds each having dimensions (5cm*2cm) were soaked in PBS for (3, 7, 14, 21 days). During soaking period the P^H was checked in every alternate day, and the PBS was replaced in every 2 days. After completion of the stipulated period of time the tensile strength of all scaffolds was tested using texture analyzer.

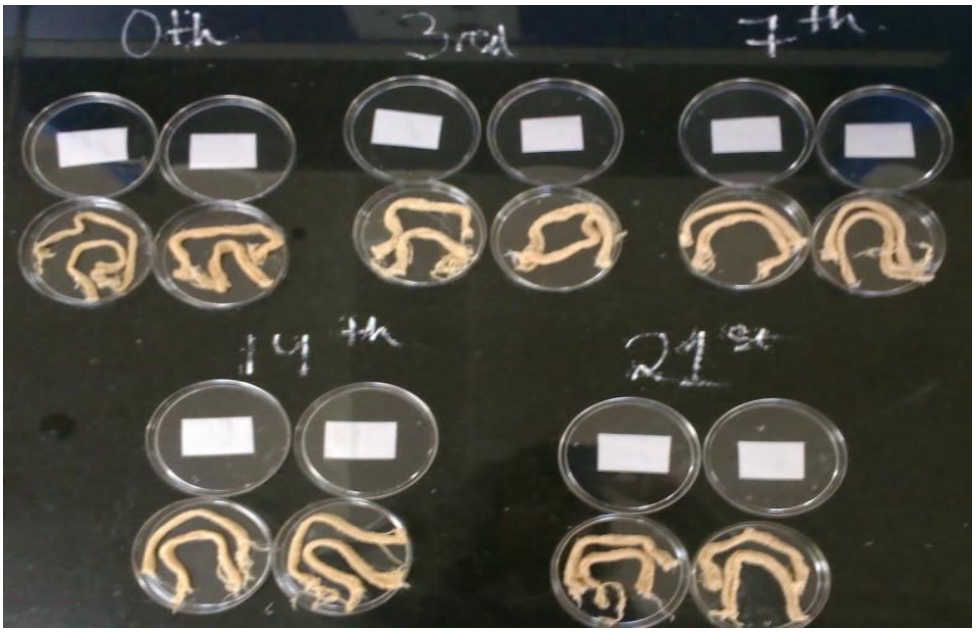


Figure 3.5 - Scaffolds in PBS medium

3.3.1 Mechanical testing of degraded scaffolds

Tensile strength of degraded scaffold was tested using TA-HDPlus Texture Analyser.

3.4 Scaffold Fabrication

3.4.1 Preparation of HA/PCL Solution

Certain amount of PCL pellets was dissolved in mixture of dichloro methane and N,N-dimethyl formamide (DCM/DMF, v/v = 85/15) to prepare 12% wt PCL DCM/DMF solution. HA was added to PCL solution, to make HA/PCL solution of different concentrations (1:1 and 0.05:1).

3.4.2 Electrospinning

5 ml of each nanocomposite solution was stirred for 24 h and the formed solutions were electrospun. In electrospinning, each of the as-prepared spinning dopes was contained in a 5 ml glass syringe, the opening end of which was connected to stainless steel needle (0.56*25mm) that was used as nozzle. The positive terminal of a variable high voltage power supply (0–15 kV) was attached to the syringe containing polymer solution whereas the ground drum covered with an aluminium foil served as opposite electrode. The distance between the syringe tip and the collector was 10 cm when the voltage applied across the

electrode reached 12 kV. After spinning for 2 h, a web of fibres accumulated on the surface of the aluminum foil was removed from the aluminium foil and dried to remove the residual solvents.

Figure 3.6 - Electrospining setup used in experiment

For Scaffold fabrication using electrospun HA/PCL solution, knitted scaffolds were kept on aluminium foil and electrospun fibres were allowed to fall on them and form nano fibres on it; thus serving as an extracellular matrix environment (ECM) for cell growth.

3.4.3 Morphological characterization of Electrospun fibres:

The morphology and structure of the fabricated knitted scaffolds having coating of electrospun fibres can be analyzed under optical scanning electron microscope. A JEOL JSM- 6480LV SEM was used in the experiment for characterization of fibers at an accelerating voltage of 15 KV. Fibre samples were cut into small pieces with the help of scissor. Small fibres were mounted with the help of carbon tape on the sample holder. Each sample was then coated with a thick layer of platinum by a JEOL JFC -1600 auto fine coater .And the operating conditions were maintained at 20 mA for 90 seconds.

3.5 FTIR Analysis

Fourier transform infrared spectroscopy (FTIR) is a technique which is used to obtain an infrared spectrum of absorption, photoconductivity, emission of a solid, liquid or gas.

Spectral data in a wide spectral range can be collected by FTIR spectrometer. This defines a significant advantage over a dispersive spectrometer which measures intensity over a narrow range of wavelengths at a time.

Figure 3.7 - FTIR Spectroscope

3.5.1 FTIR analysis of knitted fabricated HA/PCL scaffold

HA nanoparticles were taken as the reference for this analysis. HA nano particles were grinded using mortar and pestle, then the powder was used to make pellet of uniform thickness. Fabricated knitted scaffold having HA/PCL electrospun fibre, was then cut into very tiny pieces using scissor, then the scaffolds was grinded the same way as above to make the powder and hence the pellets of were taken for FTIR analysis.

3.6 Cell culture

Fibroblast cells (L929) were procured from NCCS, Pune. Procured cells were checked under microscope to check the conditions of cells. These were adherent cells from mouse.

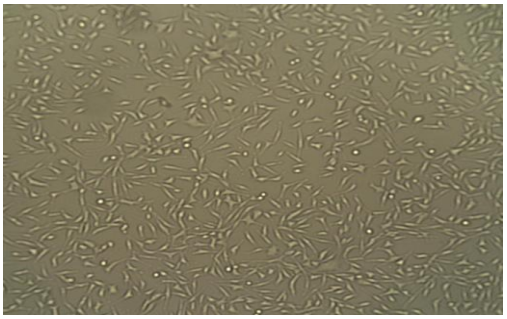


Figure 3.8 - Cultured fibroblast (*L929 cells)*

3.6.1 Preparation of DMEM

7.65gm of AT-139A-1L, 0.1825gm of L-glutamine, 0.6gm of $NaHCO_3$ was added to 400ml of autoclaved distilled water. P^H of the medium was adjusted to 7.4 (if needed by addition of 0.1N NaOH).Then the medium volume was adjusted to 500ml adding additional water .Medium was then filtered for further use.10%FBS was added to the medium before adding medium to the cells. The cells were then transferred to new flasks having 10ml of DMEM+10%FBS.Cells were observed under microscope every day to check the condition of cells. When cells were found confluent enough in the flask they were subcultured.

3.6.2 Sub-culturing of cells

The old media is first removed from the cells by pipette and discarded. The cells are then washed twice with 5ml PBS, and the waste was discarded. The cells are then trypsinised to loosen them from the culture-dish. About 1 ml of trypsin was added to all the flasks and were incubated for 5 mins. Hence, flask was checked under microscope whether cells had been detached or not. If not, flasks were again kept for more 5 mins allowing cells to detach completely. Then 5 ml of DMEM was added to flasks and all cells were taken out in a 15ml centrifuge tube after that again 5ml of DMEM was added to flasks and was pipetted to the tube. Thus the tubes were centrifuged at 2000g for 10 mins. After completion of centrifugation the pellets were pipetted using 5ml of DMEM having 10%FBS.Then extra 5ml of DMEM+10%FBS was added before incubating cells in incubator.

3.7 Cell Seeding

Scaffolds were kept in 5ml of 70% ethanol for 24 hours inside bio-safety cabinet to sterilise it. L929 Cells were seeded on sterilised scaffold and the scaffold was maintained in DMEM medium having 10% FBS. Cell adhesion test was done to check the attachment efficiency of cells on scaffold.

3.7.1 Mechanical testing of cell seeded scaffold

Tensile strength of cell seeded scaffold was tested using TA-HDPlus Texture Analyser.

3.7.2 Cell viability test

Propidium iodide (or PI) is an intercalating agent and a fluorescent molecule was used to stain cells. Propidium iodide was used as a DNA stain to evaluate cell viability or DNA content in cell cycle analysis and microscopy to visualise the nucleus and other DNA containing organelles.

3.7.3 Cell proliferation test

MTT Assay: The cells were preincubated at a concentration of 1×10^6 cells/ml in culture medium for 3 hrs at 37°C and 4 % CO_2. Then, the cells were seeded at a concentration of 5×10^4 cells/ well in 100 µl culture medium and at various concentrations (0.005-100 µg/ml) of standard Cells (dissolved in 2% DMSO (dimethylsulphoxide) solution) into microplate (96 wells having flat bottom) and was incubated for 24 hrs at 37°C and 5% CO_2. The cell proliferation is based on increasing in cell number by yellow-Orange colored propidium iodide indicator. Then, 10 µl MTT labelling mixture was added and incubated for 4 hrs at 37°C and 5% CO_2. Then 100 µl of solubilisation solution was added into each well and was incubated for overnight. The absorbance of the samples by spectrometer was measured using a microplate (ELISA-ELX-800) reader. The wavelength to measure absorbance of the dye in between 550 and 600 nm according to the filters available for the ELISA reader was used.

CHAPTER - 4

RESULTS AND DISCUSSIONS

4. Results and Discussions

4.1 SEM Image

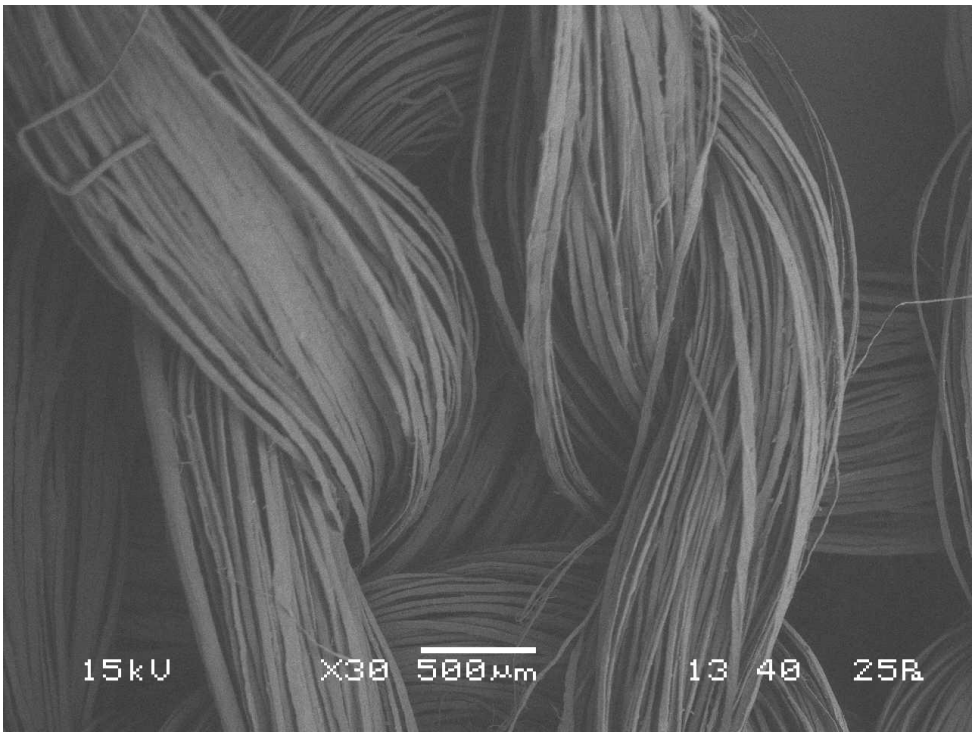


Figure 4.1 - SEM image of Knitted Silk scaffold

Pore diameter and fibre diameter were found to be 0.822 mm and 0.064 mm respectively.

4.2 Mechanical testing results of knitted scaffold

Biodegradability test:

Table 4.1 Mechanical strength of scaffolds soaked in PBS

SL. NO.	NUMBER OF DAYS	TENSILE STRENGTH (MPa)
1.	0^{th} DAY	48.12±0.18
2.	3^{rd} DAY	46.2±0.64
3.	7^{th} DAY	34.4±0.24
4.	14^{th} DAY	14.1±0.21
5.	21^{st} DAY	6.75±0.37

Gradually the tensile strength of knitted scaffold in PBS medium was found to be decreased with increasing in number of days. Decreasing in tensile strength specified that scaffolds were degraded in PBS medium.

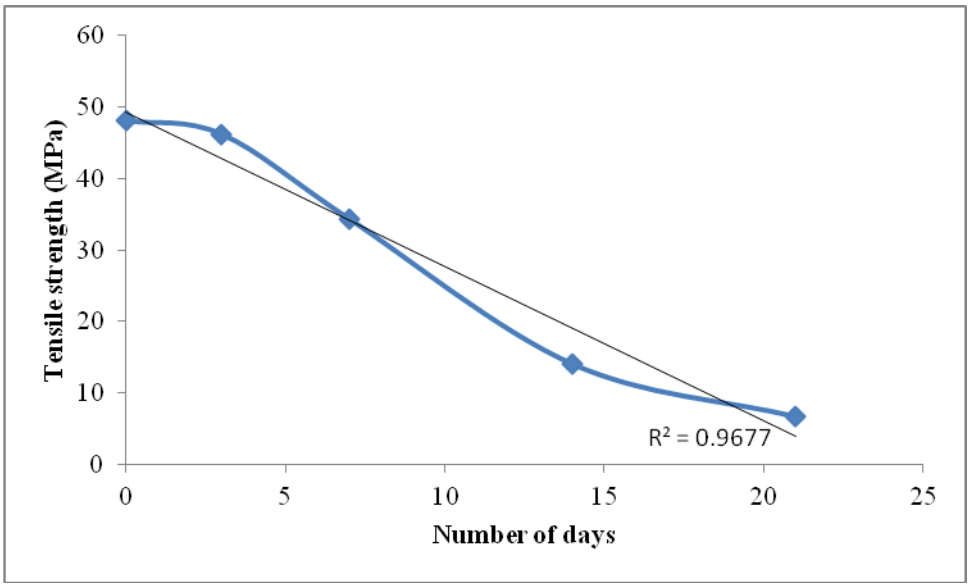


Figure 4.2 - Data plotted for degradation study of knitted scaffold in PBS

4.3 SEM Images:

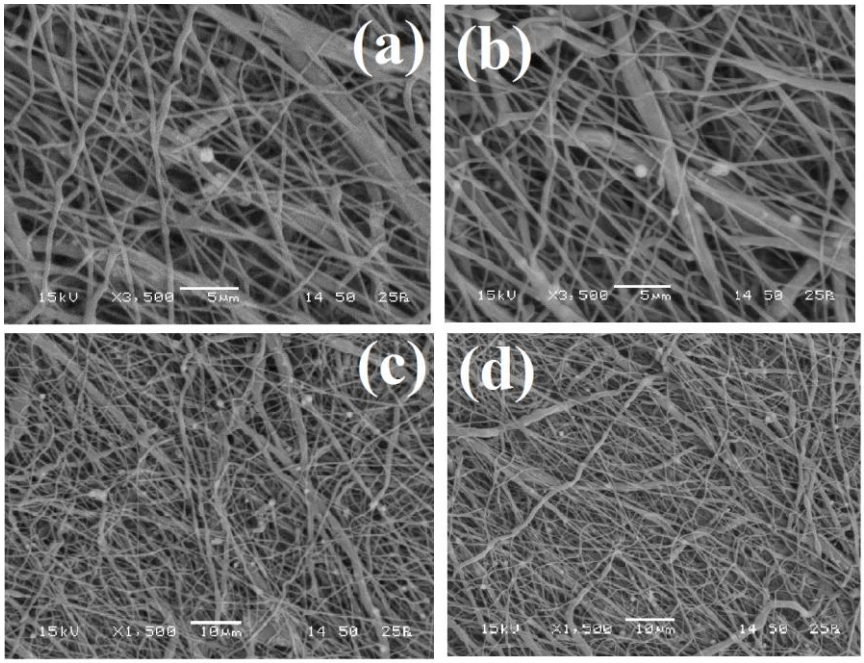


Figure 4.3 - SEM image of 1:1 ratio of HA: PCL (a) 500X (b) 1500X ,SEM image of 0.05:1 ratio of HA:PCL (c) 500X (d) 1500X.

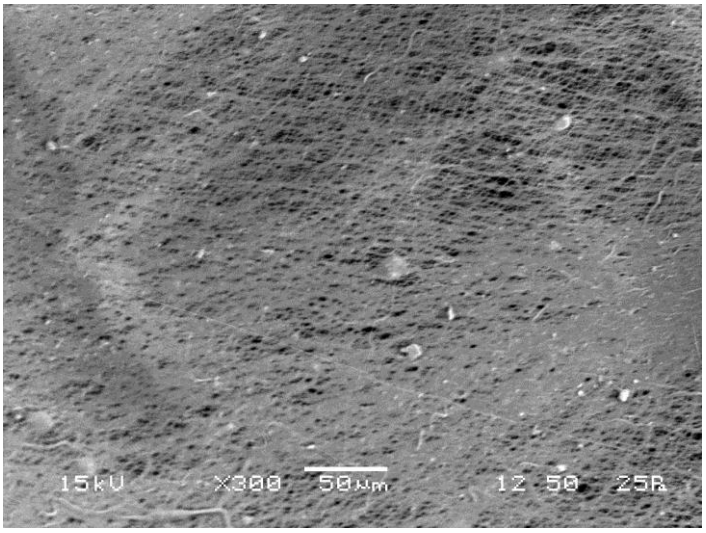


Figure 4.4 - SEM image of electrospun HA: PCL fibre on knitted silk Scaffold

SEM analysis showed random alignment of electrospun HA/PCL fibres on knitted silk scaffold. These nanofibres have diameter ranging from 335 nm to 686 nm. Surface area increases with respect to decreasing in fibre diameter. Thus, increasing in surface area provides enough potency for cell attachment and proliferation.

4.4 FTIR Results

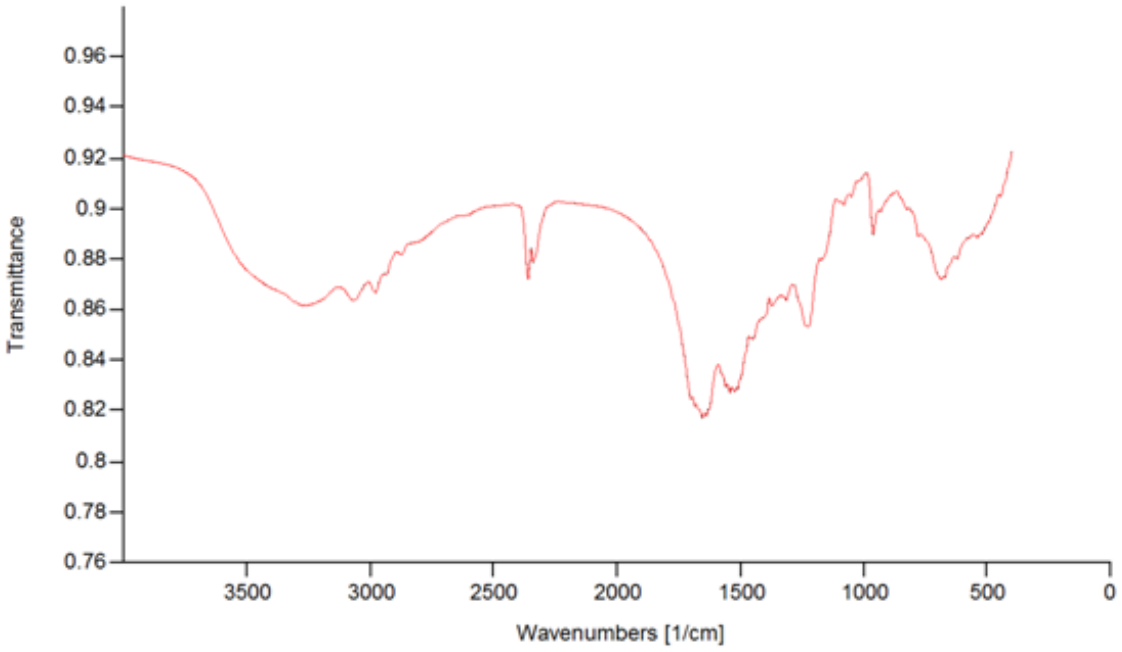


Figure 4.5 – Plot between % of transmittance vs wavenumbers for 1:1 ratio of HA: PCL.

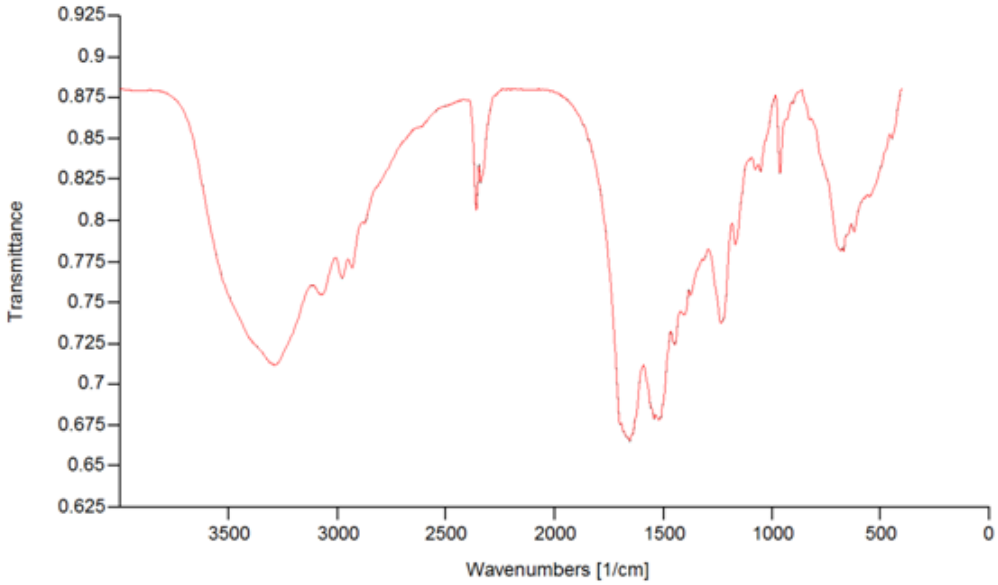


Figure 4.6 - Plot between % of transmittance vs wavenumbers for 0.05:1 ratio of HA: PCL.

The characteristic reflections of the vibrations PO_4^{3-} (950 cm^{-1}), were observed for both the coatings. These reflections indicate the rearrangement of the polyhedrons of PO_4^{3-} in the structure of the crystal. The band at (693, 679 cm^{-1}) and the sharp band at 3270 cm^{-1} are characteristic for the apatite structure and are attributed to the vibration of OH^- groups. The broad band ranging from 3287 cm^{-1} can be explained owing to O-H group stretch vibration of absorbed water.CO_3^{2-} incorporation was detected by the presence of the bands at 1422 and 1460 cm^1 (CO_3^2).

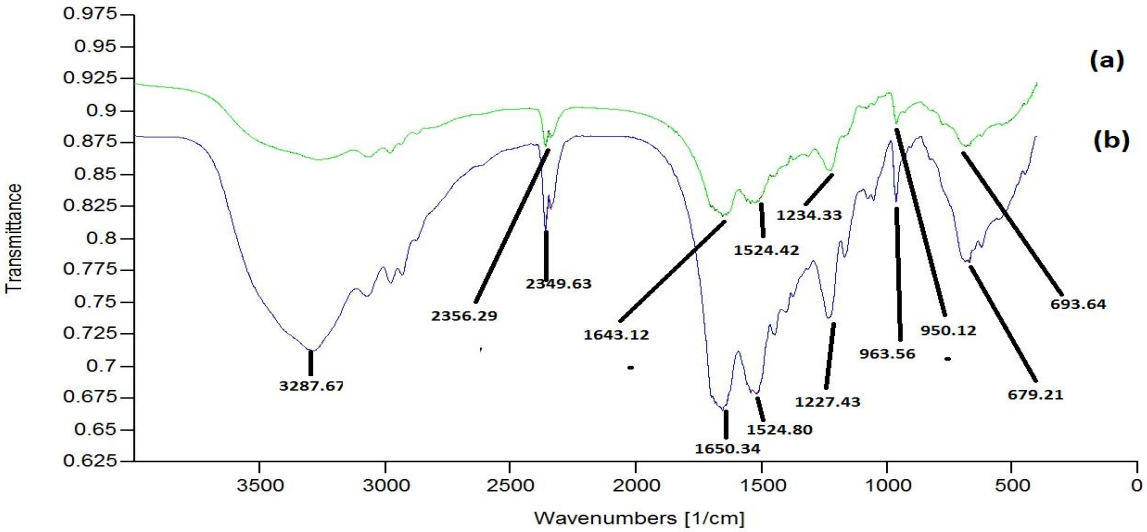


Figure 4.7 - Comparative FTIR study of HA-PCL in different concentration, (a) 1:1 ratio of HA: PCL (b) 0.05:1 ratio of HA: PCL

The C-H peaks present at 1650cm^{-1} and 2356 cm^{-1} in Electrospun PCL remained unchanged in the presence of HA. FTIR analysis has demonstrated that the electrospinning process has no effect on the functional groups present in the resulting structure. When we compare Electrospun HA & PCL Pellet the spectra reveal that there is no difference in the functional groups present after electrospinning.

4.5 Fibroblast cell (L929) culture:

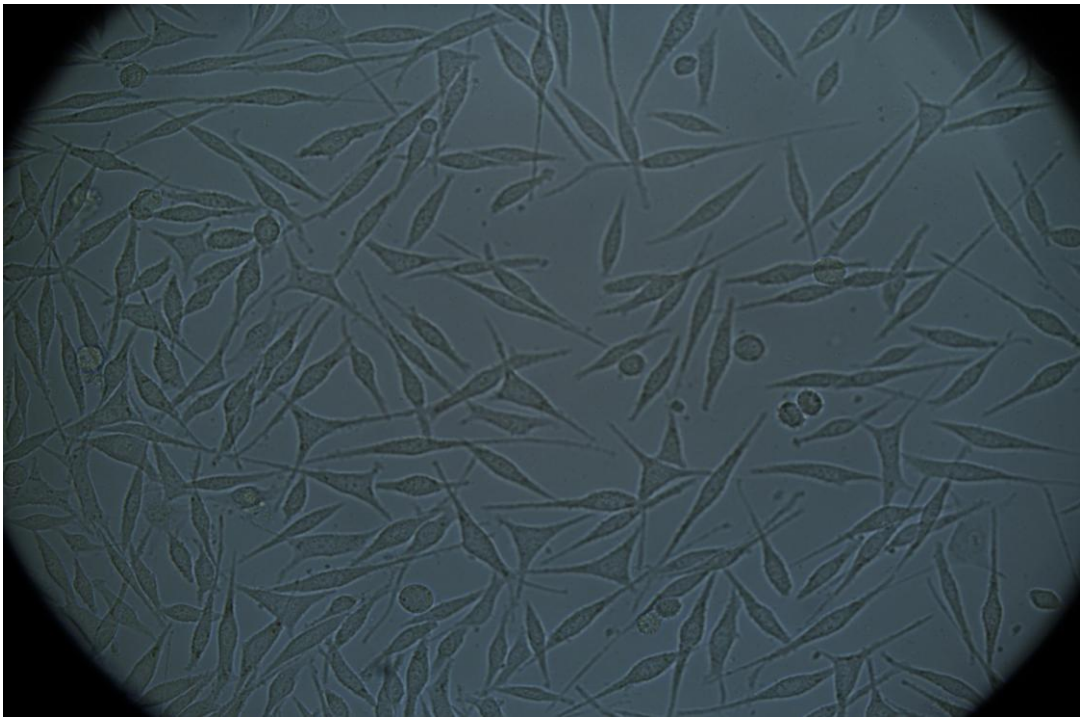


Figure 4.8 - Microscopic view of cultured adherent fibroblast (L929) cells.

4.6 Cell adhesion calculation:

Number of seeded cells = 0.6×10^6

After incubation of 24 hours,

Number of cells in medium = 0.8×10^5

Number of attached cells on scaffold = 52×10^4

% of cell adhesion =86.67%

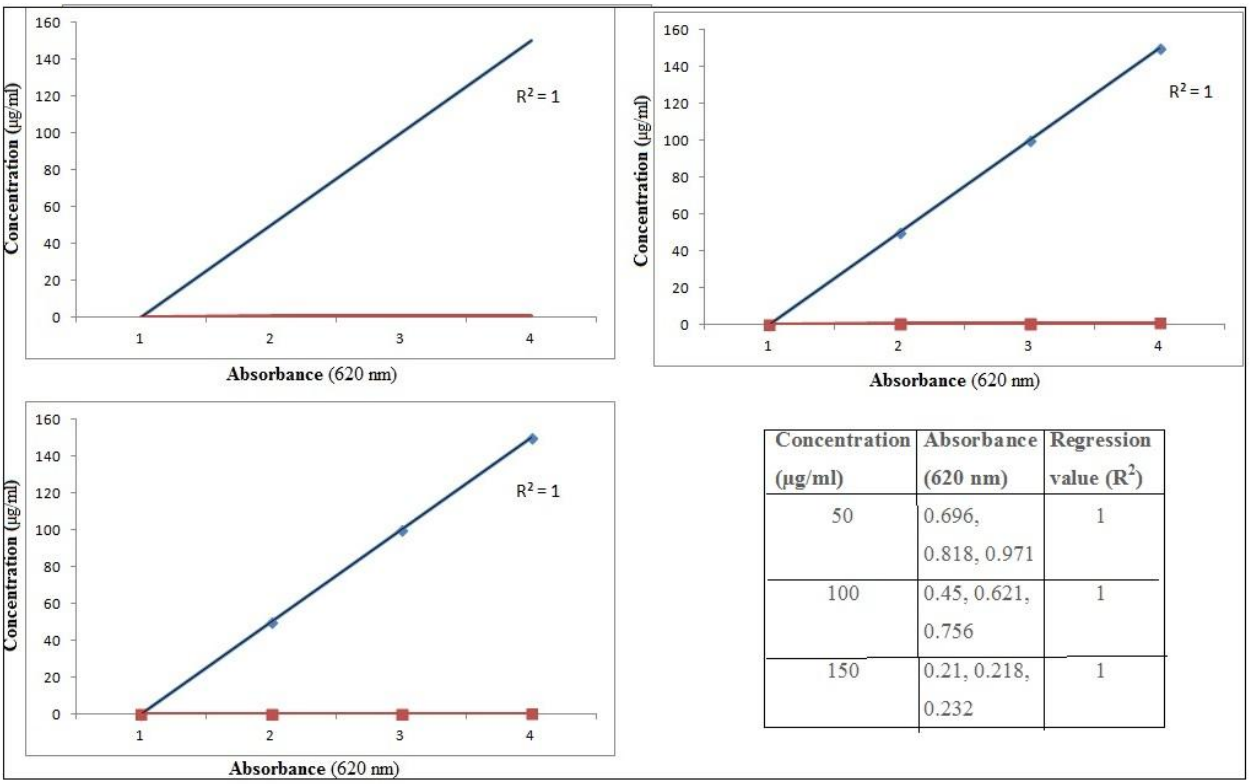
4.7 Mechanical testing result of cell seeded scaffold:

Table 4.2 - Mechanical Strength for seeded scaffold

SL. NO.	NUMBER OF DAYS	TENSILE STRENGTH (MPa)
1.	7th DAY	41.875±0.22
2.	14th DAY	46.875±0.38

4.8 MTT Assay results:

In addition, we found that the MTT assay is a more consistent model in predicting the sensitivity and specificity of cells progression obtained from allograft models. The higher sensitivity observed in assay may be in part due to extraordinary constant exposure to high cell concentrations.



Concentration (µg/ml)	Absorbance (620 nm)	Regression value (R^2)
50	0.696, 0.818, 0.971	1
100	0.45, 0.621, 0.756	1
150	0.21, 0.218, 0.232	1

Figure 4.9 - The absorption rate result of MTT assay for fibroblast cells in 24 hrs.

CHAPTER -5

CONCLUSION AND FUTURE WORK

5. Conclusion and Future work

Conclusion

Hence, a knitted silk scaffold was fabricated with electrospun HA/PCL nanoporous fibre. Mechanical strength of cell seeded scaffold was found to be 46.875MPa, which is comparable to that of bone (62MPa). The Scaffold was suitable for growth of adherent cells like fibroblast as obtained from a higher percentage (86.67%) of cell adhesion. An increment of 22% cell proliferation on 7^{th} day confirmed that the in-house fabricated scaffold is an excellent platform for cell (fibroblast *L929*) attachment, growth and proliferation. Further, a notable increase in mechanical property after cell seeding concludes a modification of fibroblast that lead to abundant extracellular matrix deposition. This result needs further confirmation by cell differentiation study.

Future work

In future, osteoblast would be used as the seeded cells in the bone compartment. Gene expression study of the seeded cells on scaffold would be analysed through real time polymerase chain reaction (RT-PCR). In addition, scaffolds would be cross linked with suitable polymers before being fabricated with electrospun HA particles.

CHAPTER - 6

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6. References

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